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A Role for Ubiquitin Binding in Bcr-Abl Transformation

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14. ABSTRACT

We have previously identified a docking site for ubiquitin in the amino-terminus of p210 BCR/ABL. In this proposal we have examined whether this association has implications for BCR/ABL signaling and transforming activity. Our approach was to map the binding site for ubiquitin in BCR/ABL and generate a binding mutant. The binding site is immediately adjacent to the GRB2 binding site, but the two binding activities are genetically separable. Although ubiquitin binding does not regulate BCR/ABL tyrosine kinase activity, the mutant can no longer interact with phosphorylated β-catenin suggesting that BCR/ABL interacts with β-catenin in a ubiquitin-dependent manner. A BCR/ABL mutant that cannot bind ubiquitin, but can still interact with Grb2, was tested for transforming activity in murine myeloid cells. The mutant can still support IL-3 independent growth in these cells indicating that some, but not all BCR/ABL activities are dependent upon this association.

15. SUBJECT TERMS

p210 BCR/ABL, ubiquitin, ubiquitin binding domain, yeast 2-hybrid mapping, animal model for chronic myelogenous leukemia

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FINAL REPORT

Introduction:

BCR/ABL is an oncogenic fusion protein that is causally associated with Philadelphia chromosome positive (Ph^+) leukemias. Although the pathogenesis of these malignancies can be directly attributed to a deregulated tyrosine kinase activity that resides within the ABL sequences, additional domains that are contained within both BCR and ABL are also required for transformation (1). Of particular note are regions within the first exon of BCR that regulate BCR/ABL kinase and transforming activity through poorly described mechanisms. In a recent study we have determined that BCR interacts with several structurally independent components of the mammalian endosomal sorting complex required for transport (ESCRT), and regulates endosome-mediated turnover of the epidermal growth factor receptor (2; EGFR). ESCRT complexes are assembled on the limiting membrane of the multivesicular body (MVB), and are responsible for sorting proteins that are targeted for lysosome-mediated degradation. While studying the role of BCR in endosomal trafficking we made the surprising observation that BCR contains a ubiquitin binding domain within its NH_2-terminus, and that the structural integrity of this site is retained in p210 BCR/ABL. Although direct binding to ubiquitin is relatively uncommon, this association has been implicated in several distinct biological processes. Importantly, recent evidence suggests that this interaction can direct a proteins own monoubiquitylation, which in turn is thought to facilitate allosteric regulation. The importance of the ubiquitin docking site for BCR/ABL transformation is unclear, and was the central issue of this proposal. The goals of the proposal were relatively straightforward; to generate a mutant of p210 BCR/ABL that no longer interacts with ubiquitin, and determine whether the mutant is impaired in signaling and/or transforming activity.

Body:

Task 1a and 1b: Map the docking site for ubiquitin by yeast 2-hybrid analysis, and construct and validate a ubiquitin binding mutant for Bcr-Abl.

In order to map the ubiquitin binding domain (UBD) we cloned full-length ubiquitin into a yeast expression vector and examined binding to BCR, and p210 BCR/ABL, by yeast 2-hybrid analysis. As shown in Figure 1A, we were able to confirm the interaction between full-length BCR and BCR/ABL by yeast 2-hybrid analysis. As indicated, a series of BCR truncation constructs were then generated and tested to further localize the UBD. This analysis revealed that there was a single UBD within BCR and BCR/ABL, and that it resides within residues 178-191. This result was reported in the last annual progress report. Since it had been previously shown by others that GRB2 binds to residue tyr177 of BCR we were concerned that our mutant may also disrupt this interaction which could confound our analysis of transforming activity. Thus, rather than immediately evaluating our mutant in transformation assays, we decided to evaluate the interaction with GRB2. For this analysis residues 178-191 were deleted in the context of p210 BCR/ABL and tested by immunoprecipitation for binding with ubiquitin (Figure 1b) and GRB2 (Figure 2a). Although the mutant was clearly impaired in ubiquitin binding, it was no longer able to interact with GRB2 suggesting that we had constructed a double mutant. Since GRB2 binding supports BCR/ABL transformation, we decided to further refine the ubiquitin binding site. Thus, a smaller deletion was constructed (Δ180-191) and evaluated for binding. This mutant is also impaired in ubiquitin binding, both in yeast and mammalian cells (Figure 1), but can still interact with Grb2 (Figure 2). This result indicated that the two binding sites are separable and provided us with a more useful mutant to evaluate the contribution of ubiquitin to BCR/ABL transformation. The need to genetically separate these two binding activities delayed, but did not impair, our ability to evaluate the mutant in the signaling and transformation assays.

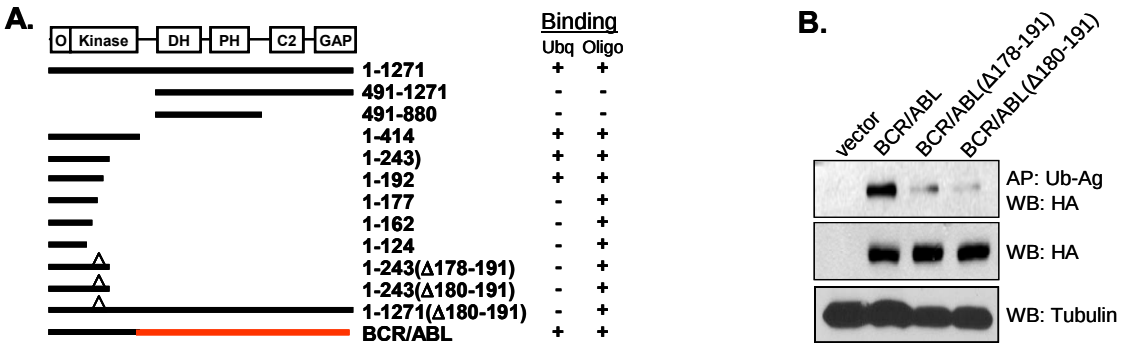


Figure 1: Ubiquitin interacts with the NH_2-terminus of BCR, and with BCR/ABL. (A) Yeast 2-hybrid analysis was used to map the UBD within the NH_2-terminus of BCR. The domain structure of the full-length BCR protein is illustrated in the upper schematic (O = oligomerization domain; kinase = serine/threonine kinase domain; DH = Dbl homology domain; PH = pleckstrin homology domain; C2 = calcium binding domain; GAP = GTPase activating protein domain) and the lines below indicate the regions of the protein included in predicted translation products of the various cDNA derivatives. All derivatives were tested for binding with full-length ubiquitin (Ubq), and with the isolated oligomerization domain of BCR (Oligo) which served as a positive control for binding. (C) 293T cells were transiently transfected with the indicated HA-tagged constructs and then lysates were collected and subjected to affinity precipitation (AP) using a ubiquitin-agarose conjugate, followed by western blot (WB) to detect BCR/ABL binding.

Task 1c: Characterize the ubiquitin binding mutant in mammalian cells.

It has been previously shown that p210 BCR/ABL autophosphorylates on Tyr-177 (within the BCR sequences), creating a docking site for GRB2 (34). As shown by co-immunoprecipitation, both p210 BCR/ABL and the smaller deletion (Δ180-191) interact with GRB2, while the larger deletion (Δ178-191) does not (Figure 2A). Retention of an interaction with a known binding partner suggests that loss of ubiquitin binding does not destabilize the BCR/ABL protein. Since the larger deletion has normal auto-kinase activity (see Figure 3A), loss of binding is probably due to the loss of residues 178 and 179 which may be required to support the GRB2 interaction. To confirm that the association with GRB2 is not impaired in the smaller mutant we also examined levels of activated ERKI/2 in the lysates. BCR/ABL has been shown to activate ERK I/2 in a GRB2-dependent manner. Consistent with this, levels of phosphorylated ERK1/2 are higher in the BCR/ABL and BCR/ABL(Δ180-191) lysates than vector lysates. Higher ERK levels were also observed in lysates that contain the larger deletion mutant which may reflect a residual interaction with GRB2. Overall our results suggest that the interaction with GRB2 does not require ubiquitin binding, and confirms that we have functionally separated these two binding activities using the smaller deletion mutant.

A recently published report suggests that the transcriptional activator β-catenin interacts with, and is stabilized by, BCR/ABL. BCR/ABL binds to phosphorylated β-catenin which stabilizes it against ubiquitin-mediated degradation. The docking site for β-catenin has been mapped to the NH_2-terminal region of BCR/ABL to an interval that includes the UBD (residues 1-202). This association is intriguing to us since β-catenin is regulated by ubiquitylation, and the docking site for BCR in β-catenin has been mapped to a single lysine residue. Thus, it is possible that this lysine is a site of ubiquitylation which is required for the interaction with BCR/ABL. To explore this possibility we also examined our lysates with antibodies that recognize total and phosphorylated β-catenin (Figure 2a). Consistent with previous reports, the level of phosphorylated β-

catenin is elevated in lysates that express BCR/ABL. However, we do not observe elevated levels of phosphorylated β-catenin in lysates that contain either of the two deletion mutants. This suggests that BCR/ABL may be binding to a ubiquitinated residue in β-catenin and preventing chain elongation and degradation. To confirm that the ubiquitin binding mutant can not stabilize phosphorylated β-catenin we probed our lysates with antibodies that recognize the different phosphorylated forms of β-catenin (Figure 2B). In each case a lower level of phosphorylated β-catenin was observed. Next we determined whether the ubiquitin binding mutant is impaired in its ability to directly interact with phosphorylated β-catenin. Thus we overexpressed BCR/ABL or the binding mutant and performed an immunoprecipitation using an antibody that recognizes phosphorylated β-catenin. Although we were readily able co-immunoprecipitate BCR/ABL with phospho-β-catenin, we were unable to IP the mutants uggesting that the interaction between BCR/ABL and β-catenin requires ubiquitination.

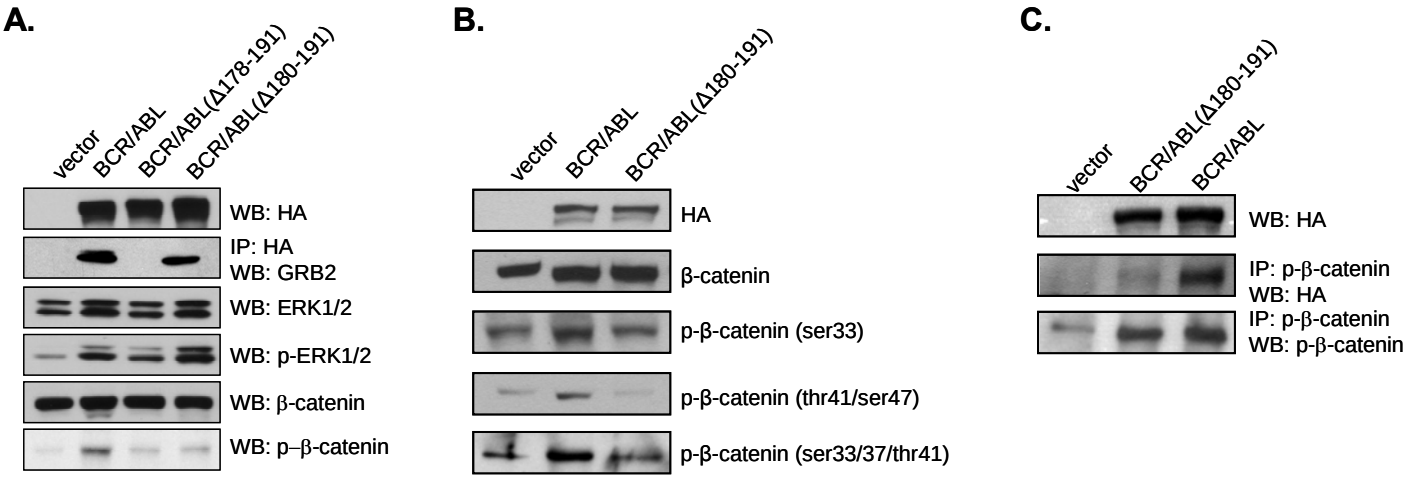


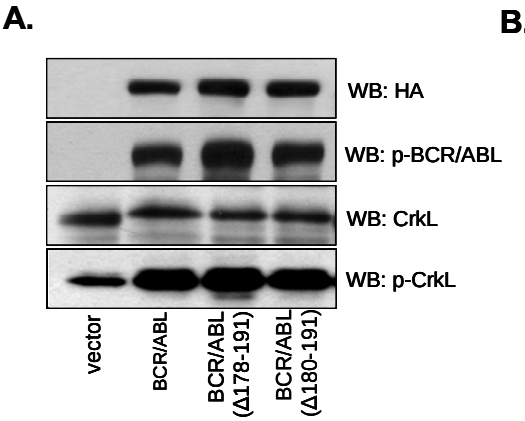
Figure 2: The ubiquitin binding mutant cannot interact with phosphorylated β-catenin. 293T cells were transiently transfected with the indicated HA-tagged constructs. Lysates were collected at 48 hours and were either immunoprecipitated (IP) or examined by western blot (WB) with the indicated antibodies. **(A)** An interaction with ubiquitin is not required for GRB2 binding, or activation of ERK 1/2, but is required for phosphor-β-catenin stabilization. Lysates were examined by western blot to determine levels of BCR/ABL expression (upper panel). Lysates were then immunoprecipitated with a BCR/ABL antibody and immunoprecipitates were examined by western blot for the presence of GRB2 (second panel). Lysates were also examined by western blot for levels of total ERK1/2 (third panel), activated ERKI/2 (fourth panel), total β-catenin (fifth panel) and phosphorylated β-catenin (bottom panel). (B) Ubiquitin binding is required to support stabilization of phospho-β-catenin. Lysates were examined by Western blot with the indicated antibodies. (C) The ubiquitin binding mutant cannot interact with phosphorylated β-catenin.

Task 2a: Test the p210 Bcr-Abl ubiquitin binding mutant in cell-based transformation assays.

It has been previously demonstrated by numerous groups that the transforming potential of BCR/ABL is dependent upon a tyrosine kinase activity that resides within the ABL sequences. Any mutation that diminishes this activity will impair transforming activity in cell- and animal-based model systems for CML. To determine

whether the tyrosine kinase activity of BCR/ABL requires ubiquitin binding, we expressed both wild-type and mutant proteins in 293T cells and performed western blots to examine the phosphorylation levels of known substrates of p210 BCR/ABL tyrosine kinase activity. As shown (Figure 3A), elevated levels of endogenous phosphorylated CRKL were observed in cells that express p210 BCR/ABL or the two deletion mutants, suggesting that the trans-kinase activity is unaffected by loss of ubiquitin binding. Next we examined the same lysates with an antibody that recognizes the Tyr-245 autophosphorylated form of p210 BCR/ABL. As shown in Figure 2A (second panel from top), the autokinase activity of p210 BCR/ABL and the two mutants is also equivalent. Thus, the overall kinase activity of BCR/ABL is not dependent upon ubiquitin binding and this activity is not regulated by ubiquitin through an allosteric mechanism.

Next we wished to determine if the mutant lacks activity in a cell-based assay for lymphoid transformation. It has been shown previously that expression of BCR/ABL is sufficient to confer interleukin-3 independent growth to the 32Dc13 murine myeloid cell line. Thus BCR/ABL and the mutant were cloned into the MSCV bicistronic retroviral vector. This vector contains GFP as position 2 which allow for FACS sorting of infected cells. Cells were sorted for GFP expression and then equal numbers were cultured in the presence or absence of IL-3 and then growth was measured at 48 hr (Figure 3B). As shown both the mutant and BCR/ABL were both able to support IL-3 independent growth in this cell type indicating that at least this parameter of transformation is not dependent upon ubiquitin binding.



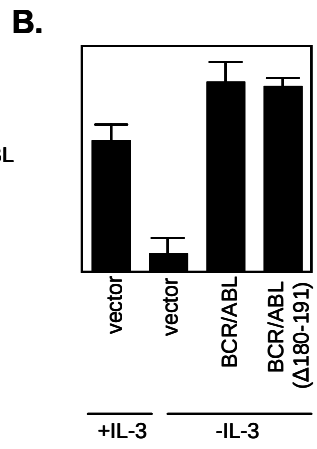


Figure 3: Ubiquitin binding does not support tyrosine kinase activity or interleukin-3 independent Growth. (A) 293T cells were transiently transfected with the indicated constructs. Lysates were collected at 48 hr and examined by Western blot with the indicated antibodies. An antibody that recognizes phosphorylated BCR/ABL was used to determine the level of autophosphorylation. (B) 32Dc13 myeloid cells were infected with the indicated MSCV retroviral constructs. Cells were FACS sorted for GFP expression and then equal numbers were plated and counted at 72 hr.

Task 2b: Test the p210 BCR/ABL ubiquitin binding mutant in a bone marrow transplantation model. Because of the unanticipated observation that the binding site for ubiquitin is immediately adjacent to the GRB2 binding site, it took longer than expected to develop a binding mutant that is uniquely impaired in its interaction with p210 BCR/ABL. Since we only recently have obtained a mutant that we are satisfied has the necessary properties, the cell based assays for transformation have been performed, but the bone marrow transplantation study has not been completed. Nonetheless, the mutant is in hand and has been successfully introduced into the bicistronic MSCV vector (see Figure 3B). The vectors have been used to transfect the Phoenix ecotropic packaging cell line and high titer retroviral stocks have been obtained for MSCV-gfp, MSCV- BCR/ABL-gfp and MSCV-BCR/ABL(Δ180-191)-gfp. We anticipate that these studies will be completed over the next 3-6 months.

Key Research Accomplishments

- We have identified the docking site for ubiquitin within the amino-terminus of BCR and p210 BCR/ABL.

- We have determined that p210 BCR/ABL contains a single docking site for ubiquitin.
- We have determined that the docking site is adjacent to, but genetically separable from the GRB2 docking site.
- We have generated and validated a ubiquitin binding mutant of p210 BCR/ABL in a mammalian expression construct.
- We have determined that ubiquitin binding supports the interaction with, and stabilization of, β-catenin. This provides a novel pathway linking BCR/ABL expression to the Wnt signaling pathway.
- We have determined that ubiquitin binding is not required to support the kinase activity of BCR/ABL through an allosteric mechanism.
- We have determined that ubiquitin binding is not required to support IL3 –independent growth in murine myeloid cells.

Reportable Outcomes

Meeting Abstracts and Publications

Chen, R., G.M. Mahon and I.P. Whitehead (2009) Exploring the role of the interaction between p210 BCR/ABL and ubiquitin in chronic myelogenous leukemia. Annual Retreat of New Jersey Commission on Cancer Research, New Brunswick, NJ.

Grants Awarded

The PI has just been awarded a grant from the Foundation of UMDNJ to continue these studies.

Personnel Receiving Pay from Research Effort

Ian P. Whitehead, Principal Investigator
Ethan Fitzpatrick, Graduate Student
Ru Chen, Graduate Student

Conclusion

During this study we have mapped the binding site for ubiquitin in BCR/ABL and constructed a mutant that lacks this binding activity. Although the mutant has normal tyrosine kinase activity it can no longer interact with and stabilize β-catenin. Since it has been previously suggested that stabilization of β-catenin contributes to p210 BCR/ABL transformation of leukemic stem cells, these observations are extremely excited. Although loss of ubiquitin binding does not appear to affect BCR/ABL transformation in cell based assays, experiments are currently underway to examine disease progression in animal models. The unexpectedly direct link between BCR/ABL and Wnt signaling is intriguing, and suggest that proteasome inhibitors should be explored as possible adjuvant treatments for CML.

References

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2. Olabisi, O. O., Mahon, G. M., Kostenko, E. V., Liu, Z., Ozer, H. L., and Whitehead, I. P. (2006) *Cancer Res* **66**(12), 6250-6257